Biological Control of Chestnut Blight: an Example of Virus-Mediated Attenuation of Fungal Pathogenesis

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INTRODUCTION

Efforts to identify determinants of virulence for plant-pathogenic fungi are complicated by the dynamic, counter-responsive nature of host-pathogen interactions and by the probability that many, rather than a few, genetic functions collectively contribute to the overall level of virulence exhibited by a given pathogen. The complexity of the pathogenic phenotype is occasionally compounded by the presence of viruses or viruslike genetic elements that can profoundly influence virulence (34, 78). This phenomenon has been regarded as a curiosity by some investigators and as a nuisance by most. However, one example of virus-mediated modulation of fungal virulence, that of transmissible hypovirulence in the chestnut blight fungus, *Cryphonectria* (*Endothia*) *parasitica*, has received more serious attention because of potential practical and experimental considerations. Interest stems primarily from the consistent correlation between the presence of viruslike double-stranded RNAs (dsRNAs) and reduced virulence (hypovirulence); the observation that the hypovirulence phenotype is transmissible to virulent strains, resulting in conversion to the hypovirulence phenotype; and the successful application of hypovirulent strains for biological control of chestnut blight. There is growing evidence that the viral elements present in hypovirulent *C. parasitica* strains alter a number of fungal processes, including host-pathogen interactions, by selectively modulating the expression of specific fungal genes and regulatory pathways. Thus, this system also provides unique opportunities for probing fungal pathogenic response mechanisms at several levels. The recent application of molecular analysis to the study of hypovirulence-associated viral RNAs has revealed considerable new information regarding the genetic organization, expression strategy, and possible origin of these genetic elements and has resulted in the identification of viral genes that are capable of conferring specific hypovirulence-associated traits. The recent introduction of a biologically active cDNA intermediate to create an artificial infectious replication cycle for a hypovirulence-associated viral RNA represents a major advancement that provides opportunities for genetic manipulations not available at the RNA level and that could have significant practical implications for efforts to restore the American chestnut. Although the primary purpose of this review is to develop a molecular perspective of transmissible hypovirulence in *C. parasitica*, attempts will also be made to provide the reader with an appreciation of the developments which led to the current view of transmissible hypovirulence and an assessment of the potential for improving and extending this form of biological control.

HISTORICAL PERSPECTIVE

Chestnut Blight and the Discovery of Transmissible Hypovirulence in *C. parasitica*

The demise of the American chestnut as a result of fungal blight early in this century is familiar to students of plant pathology as a classic example of a plant disease epidemic caused by the introduction of an exotic organism. It also remains a topic of considerable interest for segments of the general population as reflected in numerous recent articles on the subject in periodicals such as *Scientific American* (77), *National Geographic* (32), and *American Scientist* (20). Detailed accounts of the origin, progression, and consequences of the blight epidemic have been described in several excellent reviews (3, 7, 54, 71, 90). Discussion of the historical aspects of chestnut blight and the discovery of transmissible hypovirulence will be limited to the points considered necessary to prepare the reader for the main focus of this review.

The first report of chestnut blight in North America

appeared in 1905 and described symptoms on American chestnut trees located within the New York Zoological Gardens (74). The causative agent, *C. parasitica*, was apparently introduced into North America on nursery stock of resistant oriental chestnut species. The blight spread throughout the natural range of the American chestnut, which extended from Maine to Alabama and west to the Mississippi river, destroying several billion mature trees within a 50-year period (3). Since the American chestnut was the major component of the eastern hardwood forest and served as an important source of timber, tannin, and food for animal and human consumption, the ecological, economic, and even social consequences of the blight were understandably severe (80, 81).

Once established at a wound site, *C. parasitica* invades and destroys surrounding healthy tissue, forming a visible sunken canker due to necrosis and collapse of bark tissue (54, 57). Canker expansion results in the disruption of nutrient movement through the phloem and limits the ability of the host to produce new tissue as the cambium layer is damaged. Girdling of the stem results in wilting and death of the distal portion of the tree. Fortunately, the root systems of blighted trees often survive and continue to generate new shoots. However, since the shoots remain susceptible to blight, they rarely reach maturity. Consequently, the American chestnut now survives as an understory shrub where once it was the dominant species.

A brief account of chestnut blight in Europe is necessary because it was there that biological control of the disease by transmissible hypovirulence was initially observed. Blight was first reported on European chestnut (*Castanea sativa* Mill.) in 1938 near Genoa, and it spread much as it had in North America (83, 103). The observations that eventually led to the discovery of transmissible hypovirulence were first described 15 years later in a paper by the Italian plant pathologist Antonio Biraghi, in which he described several examples of trees, again in the vicinity of Genoa, that were surviving in spite of being blighted (15). Rather than the deeply indented cankers normally found on dying blight-infested trees, he noted superficial cankers that appeared to be in the process of healing. It was not until the early 1960s that the French mycologist Jean Grente and coworkers determined that the unusual symptoms described by Biraghi were not due to a resistant variety of chestnut but were a consequence of infection by altered forms of *C. parasitica* (51–53).

The French investigators found that fungal cultures isolated from the healing cankers consisted of two distinct forms. These included isolates that appeared normal in culture, i.e., produced an orange pigmentation and sporulated abundantly, and a new form that was significantly reduced in pigmentation and sporulation (51). When inoculated onto chestnut, the "white" isolates produced small, superficial cankers resembling the peripheral portions of the healing cankers from which the isolates were derived. It was this observation that led to the introduction of "hypovirulence" as a descriptive term for these new isolates. The equally significant observation made by the French workers was that the hypovirulent strains had a curative effect when inoculated onto existing cankers (53). Available evidence suggested that the application of hypovirulent strains resulted in the conversion of resident virulent strains to the hypovirulence phenotype (52). The observation that virulent strains could be converted to hypovirulence in the laboratory following anastomosis (fusion of hyphae followed by exchange of cytoplasmic material) with a hypovirulent strain

further strengthened the argument that the hypovirulence phenotype was transmissible and suggested to Grente and coworkers the involvement of a cytoplasmic genetic determinant. These combined results also set the stage for further investigations into possible applications of this transmissible form of hypovirulence as a biological control strategy.

Transmissible Hypovirulence and Biological Control

The potential of transmissible hypovirulence for biological control is perhaps best illustrated by the natural spread of hypovirulent strains and the corresponding improvement in the condition of chestnut trees in Italy (75, 99, 100). Mittempergher (75) reported in 1978 that because of the natural spread of hypovirulence during the 40 years after the blight was first detected in Italy, the disease was no longer a problem in the cultivation of chestnut in that country. After the demonstration of the curative properties of hypovirulent strains and after witnessing the apparent natural control of chestnut blight by this phenomenon in Italy during the early 1960s, Grente instituted a fairly extensive biological control program with the cooperation of the French Ministry of Agriculture in which hypovirulent strains were artificially introduced into French chestnut plantations (52). A protocol involving successive treatments of 10 cankers per hectare for 3 years followed by treatments of 5 cankers per hectare for 2 to 3 years was reported to rid plantations of virulent cankers within a 10-year period.

Reports of the successful control of chestnut blight in Europe as a result of natural dissemination (Italy) or artificial application (France) of hypovirulent *C. parasitica* strains stimulated efforts to examine whether transmissible hypovirulence might be effective in controlling blight in North America. Investigators at the Connecticut Agricultural Experiment Station confirmed that European hypovirulent strains could cure cankers incited by North American virulent strains under controlled conditions (12, 101). Native North American hypovirulent strains were subsequently found in several different geographic locations, beginning in 1976 with the isolation of a hypovirulent strain from the bark of surviving American chestnut trees located in Michigan (3, 71). Fulbright and coworkers confirmed the presence of native hypovirulent strains in various locations throughout Michigan and provided evidence for ongoing biological control (46, 47). However, initial attempts to control blight in the eastern forest ecosystem by the artificial introduction of hypovirulent strains provided less encouraging results, which have been discussed in detail elsewhere (3, 54, 71). Although clear evidence for the conversion of virulent cankers was obtained, biological control by the introduced hypovirulent strains was not sustainable. In contrast to these early reports, recent results obtained from a 9-year field study in Connecticut have provided evidence for improved chestnut tree condition as a result of the sustained spread of introduced hypovirulence in a North American forest ecosystem (9).

There are numerous opinions about why biological control by introduced transmissible hypovirulence has been less successful in North America than in Europe. One clear contributing factor to the efficient spread of introduced hypovirulent strains is the vegetative incompatibility system that controls the ability of different *C. parasitica* strains to undergo anastomosis. On the basis of limited genetic analysis, Anagnostakis (2–5) estimated that between five and seven nuclear genes determine vegetative compatibility. Two strains are vegetatively compatible and are thus freely

able to undergo anastomosis if they have the same alleles at each of these loci. The ability to form viable anastomoses decreases as the number of dissimilar alleles increases. Since the hypovirulence phenotype appears to be transmitted to virulent strains not during mating (3, 4, 8, 45) but only during anastomosis with strains of closely related vegetatively compatible groups, the complexity of the vegetative compatibility structure within the fungal population would be expected to impact the dissemination and persistence of introduced hypovirulence. Consistent with this prediction, Anagnostakis et al. (11) have reported that the vegetative compatibility diversity (the number of different vegetative compatibility types) was significantly higher in North American than in European *C. parasitica* populations. Additional factors that could negatively impact the spread and persistence of hypovirulence include reduced fitness of hypovirulent strains in specific ecosystems and reduced levels of sporulation exhibited by many hypovirulent strains (42). The importance of identifying specific factors that limit transmissible hypovirulence as an effective biological control strategy is further discussed in a later section of this review.

The first clear indication of the physical nature of the cytoplasmic genetic element responsible for transmissible hypovirulence was provided in a report by Day et al. (35) in which several hypovirulent strains were shown to harbor dsRNA molecules not present in virulent *C. parasitica* strains. It was subsequently shown that the conversion of a virulent strain to the hypovirulence phenotype following anastomosis with a hypovirulent strain was coincidental with transmission of dsRNAs (10). Fulbright (44) reported that the elimination of dsRNA from a hypovirulent strain following treatment with cycloheximide was accompanied by restoration of the virulence phenotype. Numerous additional studies have provided a preponderance of correlative evidence to suggest that the determinants responsible for the hypovirulence phenotype consist of cytoplasmically replicating genetic elements composed of dsRNA.

Since most mycoviruses have genomes composed of dsRNA (19, 68), several studies were initiated to isolate virus particles from hypovirulent *C. parasitica* strains. However, available evidence indicates that the hypovirulence-associated dsRNAs are unencapsidated and are associated with membraneous vesicles (38, 55). Like mycoviruses, hypovirulence-associated dsRNAs are not infectious in the classical sense; i.e., cell-free preparations are not infectious when applied to fungal hyphae or spheroplasts. Transmission from strain to strain occurs only following anastomosis. The inability of these elements to initiate an infection by an extracellular route has hampered efforts to rigorously demonstrate a direct cause-and-effect relationship.

Surveys of dsRNAs associated with different hypovirulent strains revealed considerable variations in the concentration, number, and size of dsRNA components (10, 39, 42). Variation in the degree of hypovirulence and associated traits such as reduced pigmentation or suppressed sporulation was also observed for different dsRNA-containing hypovirulent strains (42). However, it is important to note that the traits exhibited by a particular hypovirulent strain appeared to be independent of the nuclear genetic background, since similar traits were observed when the same dsRNA species were transmitted into different virulent strains following anastomosis (42). Although descriptive and correlative studies were extremely valuable in identifying a role for dsRNAs in hypovirulence, it was clear by the mid-1980s that a mechanistic understanding of transmissible hypovirulence required the ability to manipulate hypovirulence-associated
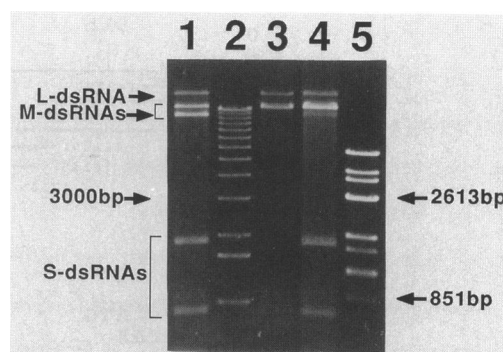

FIG. 1. Gel banding patterns of dsRNAs present in hypovirulent *C. parasitica* EP713. dsRNAs prepared from three different single conidial isolates which were cultured for different lengths of time over a 6-month period were separated on a 1% agarose gel and detected under UV light after being stained with ethidium bromide (lanes 1, 3, and 4). Lane 2 contains the 1-kb DNA ladder as size markers, and lane 5 contains the dsRNA genome of wound tumor virus. The sizes of representative wound tumor virus genomic segments are indicated at the right, and the migration position of the 3-kb DNA marker is indicated at the left. Also indicated at the left are the migration positions of L-dsRNA (12.7 kb); the M-dsRNAs, which range in size from 8 to 10 kb; and the S-dsRNAs, which range in size from 0.6 to 1.7 kb. Reprinted with permission from *EMBO Journal* (93).

dsRNAs. This, in turn, was dependent on progress in determining basic structural and functional properties of these genetic elements.

MOLECULAR CHARACTERIZATION OF HYPOVIRULENCE-ASSOCIATED VIRAL RNAs

Consistent with the observed variations in the size, number, and concentration of hypovirulence-associated dsRNAs, results of molecular hybridization studies indicated considerable diversity in sequence similarity among these genetic elements (70, 82). The situation was further complicated by the observations that most hypovirulent strains contained multiple species of dsRNAs (10, 39, 42) and that the number and concentration of individual dsRNA species harbored by a particular hypovirulent strain could vary as the fungus was cultured (10, 93). Although the picture remains complex, recent application of molecular analysis has brought some order to the confusion.

Genetic Organization

The current view of the genetic organization and basic expression strategy of hypovirulence-associated dsRNAs is drawn principally from detailed characterizations of the dsRNAs present in a single hypovirulent *C. parasitica* strain, the European-derived hypovirulent strain EP713 (29, 60, 87, 93). As indicated in Fig. 1, this strain harbors a number of dsRNA components that were originally given the designations L-dsRNA, M-dsRNAs, and S-dsRNAs on the basis of their relative migration rates on gels and corresponding estimated sizes (60, 93). It is also clear from inspection of Fig. 1 that the gel-banding pattern can change as the fungus is cultured. Only L-dsRNA remains stable in size and concentration with time of culturing, whereas the number and concentrations of individual M- and S-dsRNA species vary.

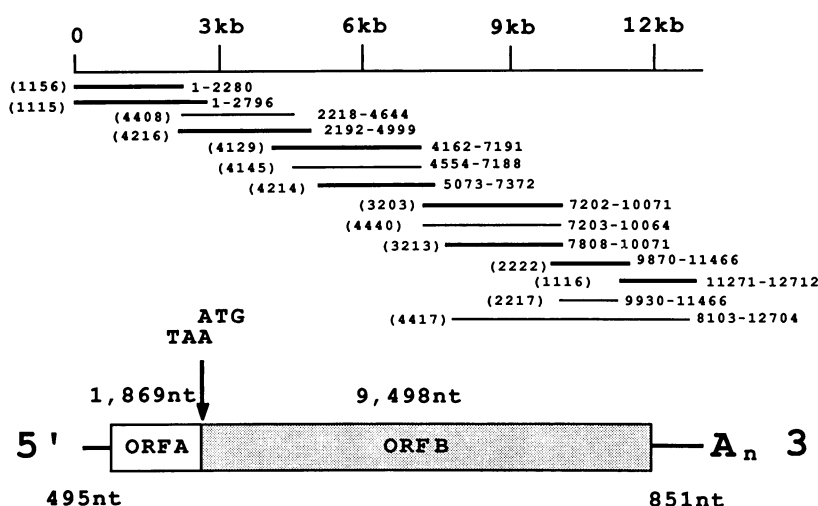The first insights into the general structural organization of

FIG. 2. Cloning and basic organization of L-dsRNA from hypovirulent *C. parasitica* EP713. A collection of overlapping cDNA clones that spanned the entire length of L-dsRNA is shown at the top of the figure. Clone designations are indicated to the left of each line, and the map coordinates covered by each clone are indicated to the right of each line. Clones represented by the thick lines were sequenced on both strands, whereas clones represented by the thin lines were sequenced on only one strand. The general organization of the coding or sense strand of L-dsRNA is indicated at the bottom of the figure and includes a 495-nt noncoding leader sequence, the 1,869-nt ORFA, the pentanucleotide 5'-UAAUG-3' located at the ORFA/ORFB junction, the 9,498-nt ORFB, an 851-nt noncoding 3'-terminal domain, and the 3' poly(A) tail. Reprinted with permission from *EMBO Journal* (93).

the dsRNA genetic elements present in strain EP713 was provided by Hiremath et al. (60) as a result of direct analysis of the terminal domains of individual dsRNA species. One terminus of each dsRNA species was found to consist of a 3' poly(A) tract base paired to a 5' poly(U) tract while the other end was found to contain a consensus 28-nucleotide 3'-terminal sequence. Tartaglia et al. (98) reported a similar organization for the terminal domains of the dsRNAs associated with the North American hypovirulent strain Grand Haven 2 (GH2), providing the first indication of common structural properties for different hypovirulence-associated dsRNAs. Using the structural information obtained from direct analysis of the EP713 dsRNAs as an aid, Rae et al. (87) were able to generate large cDNA clones corresponding to the terminal domains and were subsequently able to identify the first hypovirulence-associated dsRNA-encoded protein, designated p29. Molecular hybridization analysis had previously shown that in both strains EP713 and GH2, the poly(A)-containing dsRNA strand was present in both a single-stranded RNA (ssRNA) form and a dsRNA form whereas the poly(U)-containing strand was present exclusively in a dsRNA form (60, 98), suggesting that the poly(A) ssRNA may function as mRNA. Subsequent sequence analysis of cDNA clones spanning the entire EP713 L-dsRNA molecule confirmed that only one strand, the poly(A) strand, contained coding domains of significant size (93). As illustrated by the cartoon presented in Fig. 2, results of that study concluded that L-dsRNA is 12,712 bp long, excluding the poly(A)-poly(U) domain. Two open reading frames (ORFs) designated ORFA and ORFB, extending 622 and 3,165 codons, respectively, were found within the poly(A)-containing coding strand. They are preceded by a 495-nucleotide (nt) 5' noncoding leader sequence and a 851-nt 3' noncoding domain.

In a paper accompanying the above report, Shapira et al. (92) confirmed the earlier proposal that the M- and S-dsRNA components were derived from L-dsRNA as a result of internal deletion events. Reverse transcriptase-coupled

polymerase chain reaction mapping revealed that the M-dsRNAs consisted of a family of internally deleted forms of L-dsRNA that had retained approximately 3.5 kb of the terminal portions (Fig. 3), i.e., the deletion breakpoints were located within the internal regions of the L-dsRNA se-
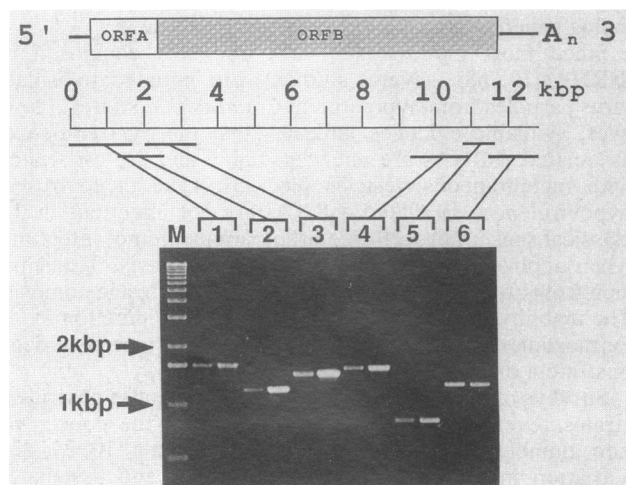

FIG. 3. Comparative polymerase chain reaction mapping of the terminal portions of M-dsRNA and L-dsRNA. Primer pairs spanning the terminal portions of the L-dsRNA sequence map were used to amplify specific portions of cDNA generated from purified L-dsRNA and M-dsRNAs. The portions of the L-dsRNA map specified by each primer pair are indicated by the horizontal bars. Amplification products were analyzed by 1% agarose gel electrophoresis, as indicated at the bottom of the figure. The well marked M received the 1-kb DNA ladder as size markers. Results were consistent with earlier proposals (87) that the M-dsRNAs consist of a family of internally deleted forms of L-dsRNA that have retained approximately 3.5 kb of the terminal portions. Reprinted with permission from *EMBO Journal* (92).
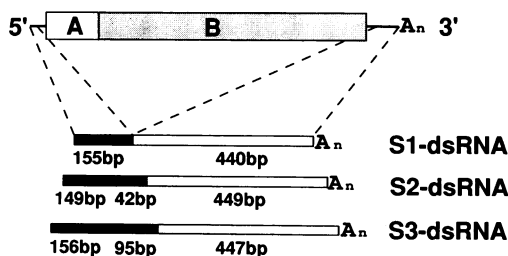
FIG. 4. Schematic representation of cDNA clones of several S-dsRNAs associated with hypovirulent *C. parasitica* EP713. Sequence analysis of a collection of S-dsRNA cDNA clones confirmed that each S-dsRNA contained both of the L-dsRNA terminal domains. For example, S1-dsRNA was found to contain 155 bp of the terminus corresponding to the 5' end of the coding strand [5'-(+)] and 440 bp of the other terminus [3'-(+)], excluding the poly(A): poly(U) domain, and contained a single deletion breakpoint. S2-dsRNA and S3-dsRNA contained 149 and 156 bp, respectively, of the 5'-(+) terminus and 447 and 449 bp, respectively, of the 3'-(+) terminus. However, in addition, each of these RNAs contained a 42-bp sequence between the recognizable L-dsRNA deletion breakpoints that was unrelated to any L-dsRNA sequences. In S3-dsRNA, the 42-bp sequence was flanked by additional nonhomologous sequences: 23 bp on the 5'-(+) side and 30 bp on the 3'-(+) side.

quence. Direct characterization of the GH2 dsRNAs had also indicated that a 3.5-kb dsRNA species was derived from the largest species by an internal deletion event (98). These results suggest that the generation of internally deleted defective RNA molecules is a common feature of hypovirulence-associated dsRNA replication which contributes significantly to the observed complex gel banding patterns. Given the small size of the S-dsRNA species (600 to 800 bp) and their ability to efficiently replicate in the presence of L-dsRNA, it was reasoned that characterization of these dsRNAs would provide a first approximation of the location of *cis*-regulatory elements involved in the replication of these dsRNA genetic elements. Sequence analysis of cloned cDNA copies of several S-dsRNA species revealed that they retained only the terminal noncoding regions of L-dsRNA: ~150 bp of the terminus corresponding to the 5' end of the coding strand and ~450 bp of the other terminus (Fig. 4). In practical terms, one could imagine that such conserved terminal domains could serve as replication signals for amplification of foreign genetic information introduced into hypovirulent *C. parasitica* strains, thus allowing the development of a dsRNA-based expression system.

Unexpectedly, two of the prominent S-dsRNA species were found to contain additional sequence information that appeared unrelated to the L-dsRNA sequence (Fig. 4). One species contained a 42-bp nonhomologous sequence inserted between the recognizable L-dsRNA deletion breakpoints, and the second species contained the identical 42-bp sequence flanked by an additional 23 bp on one side and 30 bp on the other. Although these nonhomologous domains could represent severely rearranged L-dsRNA sequences, the fact that an exact copy of the 42-bp oligomer found in one S-dsRNA species was also present within the breakpoints of a second S-dsRNA species suggests an origin other than L-dsRNA for these sequences. An alternative explanation offered by Shapira et al. (92) was that the nonhomologous sequences represent cellular RNA that was introduced as a result of a recombination event between L-dsRNA and cellular RNAs. The implications of the apparent high fre-

quency of internal deletion events and possible recombination with cellular RNAs for the evolution and origin of hypovirulence-associated dsRNAs will be examined in a later section.

Sequence information for dsRNAs associated with hypovirulent *C. parasitica* strains other than EP713 have recently become available. Hillman et al. (59) reported the generation of a cDNA library representing the entire single large dsRNA (~12.5 kb) present in a North American hypovirulent isolate designated NB58. Molecular hybridization experiments with the mapped cDNA clones indicated sequence similarity between NB58 and the European-derived EP713 dsRNA. Sequence analysis of cDNA clones corresponding to the terminal domains of NB58 dsRNA confirmed the hybridization results and revealed an organization similar to that of EP713 L-dsRNA. These results and the absence of cross-hybridization between NB58 dsRNA sequences and any other North American hypovirulence-associated dsRNAs prompted Hillman et al. (59) to suggest that NB58 may be related to European-derived hypovirulent strains that were released in the northeastern United States during the 1970s in early attempts at hypovirulence-mediated biological control in North America. As indicated above, Tartaglia et al. (98) had identified similarities in the gross structural properties between EP713 dsRNAs and the dsRNAs present in the North American hypovirulent strain GH2. Although GH2 and EP713 dsRNAs do not cross-hybridize, recent sequence analyses of cDNA clones generated from GH2 dsRNA have identified deduced amino acid sequence motifs that clearly correspond to similar motifs within coding domains found in EP713 L-dsRNA (40). Although all the hypovirulence-associated dsRNAs that have been subjected to molecular analysis appear to be related and many hypovirulent *C. parasitica* strains contain a dsRNA species the approximate size of the EP713 L-dsRNA, it is important that the reader not be led to understand that all hypovirulence-associated dsRNAs conform to the genetic organization typified by EP713 L-dsRNA. For example, several hypovirulent strains contain dsRNAs consisting of two or more prominent unrelated dsRNA species in the range of 2.5 to 7 kb, suggesting an organization quite different from that exhibited by EP713 L-dsRNA (82). Determination of the structural properties of these additional hypovirulence-associated dsRNAs will be of considerable interest.

Expression Strategy

Consistent with the viruslike genetic organization of the EP713 L-dsRNA, the basic mechanisms involved in expression of the encoded gene products are also similar to those used by viruses (Fig. 5). As first noted by Rae et al. (87), the 5' noncoding leader sequence of the L-dsRNA coding strand contains six potential translation initiation codons, each of which is followed closely by one or two termination codons. As would be predicted by the work of Kozak (64) and Mueller and Hinnebusch (76), removal of these six minicistrons resulted in a 10-fold increase in the level of in vitro expression of downstream sequences (87). The role of the 5' leader in the in vivo expression of L-dsRNA gene products has not yet been thoroughly elucidated. However, the coding strand of NB58 dsRNA was also shown to have a long 5' noncoding leader (487 nt) that contains nine minicistrons, suggesting a possible functional role for this domain (59).

As is the case for many viral systems, proteolytic processing has been found to play a fundamental role in the expression of the two coding domains present in L-dsRNA.
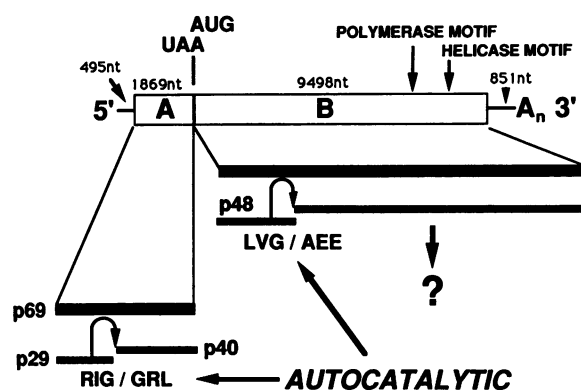
FIG. 5. Current view of the basic gene expression strategy of L-dsRNA present in hypovirulent *C. parasitica* EP713. The 495-nt noncoding leader seuqence preceding ORFA contains six potential initiation codons, each of which is followed closely by one or two termination codons. Removal of the leader results in a 10-fold stimulation in the in vitro translation of downstream coding regions (29, 87). ORFA (622 codons) encodes two polypeptides, p29 and p40, that are released from polyprotein p69 by an autocatalytic event mediated by p29. Cleavage occurs between Gly-248 and Gly-249 during translation and is dependent on the essential Cys-162 and His-215 (28, 29). The junction between ORFA and ORFB consists of the sequence 5'-UAAUG-3'. Translational mapping studies have indicated that the UAA portion of the pentanucleotide serves as a termination codon for ORFA, while the AUG portion is the 5'-proximal initiation codon of ORFB. Expression of ORFB also involves an autoproteolytic event in which a 48-kDa polypeptide, p48, is released from the N-terminal portion of the encoded polyprotein. In this case, cleavage occurs between Gly-418 and Ala-419 and is dependent on essential residues Cys-341 and His-388 (94). Both p29 and p48 resemble papainlike proteinases, and one proteolytic domain may have arisen as a result of a duplication of the other (28, 63, 94). Computer-assisted analysis revealed five distinct domains within the L-dsRNA coding regions that showed significant sequence similarity to previously described domains within plant potyvirus-encoded polyproteins (63). These included putative RNA-dependent RNA polymerase and RNA helicase motifs located in the approximate portions of ORFB indicated by the arrows. ORFB is followed by an 851-nt noncoding domain and a 3' poly(A) tail. Modified and reprinted with permission from *EMBO Journal* (93).

The 5'-proximal coding region, ORFA, encodes a 69-kDa polyprotein that is autocatalytically processed during in vitro translation to produce a 29-kDa protein, p29, from the amino-terminal portion and a 40 kDa protein, p40, from the carboxy-terminal domain (29). The p29 cleavage product has also been found in extracts of hypovirulent strain EP713 by Western immunoblot analysis (87). Recent studies with antisera directed against the p40 domain, however, suggest that this cleavage product undergoes additional processing in vivo (32a). The approximate location of the catalytic site involved in autoproteolysis of p69 was mapped to the carboxy-terminal one-third of p29, and the cleavage dipeptide site was identified as Gly-248/Gly-249 by microsequence analysis (29). Subsequent amino acid substitution analysis confirmed the position of the cleavage dipeptide and revealed that p29 residues Cys-162 and His-215 were essential for autocatalytic cleavage (28).

Autoproteolysis was also found to be involved in the expression of ORFB (93). A 48-kDa polypeptide, designated p48, was shown to be released from the amino-terminal portion of the ORFB-encoded polyprotein during in vitro translation. Using site-directed mutagenesis and bacterial expression systems, Shapira and Nuss (94) demonstrated

that residues Cys-341 and His-388 were essential for autoproteolysis and identified Gly-418/Ala-419 as the cleavage dipeptide. Since ORFB has the capacity to encode a ~345-kDa polyprotein, it is a likely substrate for additional processing. There are numerous examples of virus-encoded proteinases that cleave in *trans* once they are autocatalytically released from a polyprotein (65, 102). However, repeated attempts to demonstrate cleavage in *trans* by both p29 and p48 have been unsuccessful.

The junction between ORFA and ORFB consists of the unusual pentanucleotide 5'-UAAUG-3', in which the 5'-proximal residues, UAA, have been shown to serve as the termination codon of ORFA (29, 93) and the 3'-proximal residues, AUG, represent the first potential initiation codon of ORFB (Fig. 5). Interestingly, the identical pentanucleotide sequence has been reported to function in the translation of tandem cistrons found within RNA segment 7 of influenza B virus (61). Results of mutational analyses of the pentanucleotide and surrounding residues suggested that translation of the bicistronic mRNA derived from the influenza genomic RNA segment involved a termination-reinitiation scheme. It will be interesting to determine whether a similar mechanism operates during translation of the two coding domains of L-dsRNA or whether ORFB expression depends on an alternative strategy such as the generation of a subgenomic transcript.

There remain many questions concerning the precise details of posttranslational processing of the L-dsRNA-encoded polyproteins and the nature of mechanisms involved in the regulation of L-dsRNA replication, transcription, and translation. However, it is clear that L-dsRNA resembles viral RNA genomes in terms of genetic organization and basic expression strategy. This similarity prompted Shapira et al. (93) to propose that L-dsRNA be considered the equivalent of a viral genome or replicative intermediate and to suggest hypovirulence-associated virus (HAV) as a tentative descriptive term for this class of genetic elements. In considering the merits of this proposal, it is important to note that the dsRNAs associated with hypovirulent strains differ from classical viruses in that they appear not to be packaged within discrete particles and are not infectious by an extracellular route. Rather, they are reportedly associated with pleomorphic membraneous vesicles (38, 55) and are transmitted to other *C. parasitica* strains only by cytoplasmic exchange during anastomosis. On the other hand, results of recent comparative sequence analysis are entirely consistent with a viral origin for these genetic elements (63).

Origin and Evolution

The first indication of sequence similarity between L-dsRNA coding domains and virus-encoded proteins surfaced during comparisons of the amino acid sequence and properties of p29 with those of virus-encoded proteinases. Carrington et al. (22) had reported that the proteinase HC-Pro, encoded by tobacco etch virus, a plant potyvirus, released itself from a polyprotein by autocatalytic cleavage at a glycylglycine dipeptide. Moreover, Oh and Carrington (79) had shown by site-directed mutagenesis that Cys-649 and His-722 of HC-Pro were essential for proteolytic activity. Alignment of the putative cleavage sites and upstream cysteine and histidine residues for p29 and HC-Pro revealed a striking similarity between the sequences surrounding the essential cysteine and histidine residues of the two proteinases and in the spacing of these residues relative to the respective cleavage sites (29). As indicated in Fig. 6, a very

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                        *      *
MCP        QGGQCGSCWAFS- 129 -NLDHGVLL
ACTINIDIN  QGECGGCWAFS-  129 -AVDHAIVI
DCP        QGQCGSCWSFS-  139 -SLDHGILI
PAPAIN     QGSCGSCWAFS-  128 -KVDHAVAA
                                                    1  1'
           *             *
TEV-HC-Pro  IANEGMCYMNI- 64 -KTMHVLDS- 31 -KTYNVG GMNR
TVMV-HC-Pro IAKEGMCYINI- 64 -KTIHVVDS- 31 -AQYKVG GLVY
HAV-P29     QFGQGMCYLSA- 44 -HVYHVVVD- 23 -PLARIG GRLN
HAV-P48     PVEEGRCFELL- 38 -QCVHIVAG- 20 -PDILVG AEEG
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FIG. 6. Alignment of the cysteine and histidine residues and the cleavage sites for p48, p29, proteinase HC-Pro (encoded by two potyviruses), and the corresponding domains of several papainlike proteinases of cellular origin. The essential residues are indicated by *, and conserved residues are boxed. The arrow indicates the autocatalytic cleavage residues for the virus-encoded proteinases. The numbers of amino acid residues between essential residues and cleavage sites are also indicated for the viral proteinases. Abbreviations: MCP, mouse cysteine proteinase; DCP, *Dictyostelium* cysteine proteinase; TEV, tobacco etch virus; TVMV, tobacco vein mottling virus. Modified and reprinted with permission from *Journal of Biological Chemistry* (94).
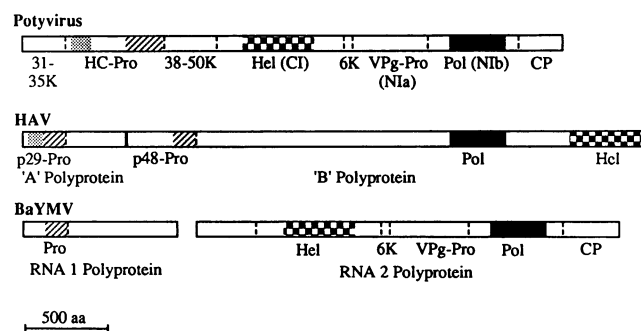
FIG. 7. Schematic comparison of the organization of the polyproteins of HAV, potyviruses, and barley yellow mosaic virus (BaYMV). Related domains are highlighted by identical shading, and proteolytic cleavage sites are denoted by dashed lines. The termination codon of HAV ORFA is indicated by a solid vertical line. Abbreviations: Pol, RNA polymerase; Hel, helicase; Pro, proteinase; CP, capsid protein; aa, amino acid. Reprinted with permission from *Proceedings of the National Academy of Sciences of the United States of America* (63).

similar pattern is also conserved in p48. The sequences around the essential Cys residue of each of the viral proteinases consists of a conserved Gly residue located two positions toward the amino-terminal side and an adjacent aromatic amino acid residue on the carboxy-terminal side. A conserved stretch of hydrophobic amino acids is also evident adjacent to the essential histidine on the carboxy-terminal side. The resemblance of these motifs to the catalytic domains of cellular papainlike proteinases prompted the proposal that these viral proteinases be placed within the cysteine proteinase superfamily (79, 94). The cleavage sites of the three viral proteinases also share some similarities that include a requirement for a glycine residue at the P1 position, a residue containing a small uncharged R group at the P1' position, and a valine or isoleucine residue at the P2 position (23, 94). The similarity in the distances between the two essential residues and the cleavage sites for the three proteinases is also notable. In this regard, the similarities between p29 and p48, including their relative locations within the amino-terminal portions of the two L-dsRNA-encoded polyproteins, led Shapira and Nuss (94) to speculate that the two respective coding regions are products of a gene duplication event.

Shapira et al. (93) reported an additional conserved motif located within the carboxy-terminal portion of ORFB that corresponded to an RNA helicase domain found within the potyvirus cylindrical inclusion protein. These early observations, resulting initially from simple visual inspection of deduced sequence information, prompted Koonin et al. (63) to conduct a detailed, computer-assisted analysis of the L-dsRNA coding domains. Surprisingly, five distinct domains with significant sequence similarity to previously described conserved domains within potyvirus polyproteins were identified. In addition to the above-mentioned proteinases and RNA helicase motifs, these domains of similarity included a putative RNA-dependent RNA polymerase and a cysteine-rich domain of unknown function similar to the N-terminal portion of the HC-Pro proteinase. Phylogenetic trees derived from the alignment of the L-dsRNA polymerase domain with all known viral RNA-dependent RNA polymerase sequences indicated a much closer similarity to polymerases encoded by ssRNA viruses than to polymerases encoded by dsRNA viruses and strongly suggested a common ancestry for L-dsRNA, the plant potyviruses, and

the closely related barley yellow mosaic virus (BaYMV). This proposal is interesting in light of earlier speculation by Tartaglia et al. (98) that hypovirulence-associated dsRNAs are analogous to the replicative form of an ancestral ssRNA virus.

Since the organization of the conserved domains within the polyproteins encoded by L-dsRNA, potyviruses and BaYMV differ significantly (Fig. 7), an evolutionary pathway from a common ancestor would have had to involve extensive rearrangement. Assuming that the ancestral genomic RNA was single stranded and potylike in organization, Koonin et al. (63) suggested a scenario for L-dsRNA evolution that included the following events: (i) gene duplications to form the p29 and p48 proteinase domains, (ii) transposition of the helicase and polymerase domains, (iii) deletion of certain ancestral genes such as the capsid gene (L-dsRNA is not encapsidated), and (iv) emergence of a termination codon to separate ORFA and ORFB. Although it is speculative, several aspects of the scenario are intriguing. For example, given the absence of restrictions on movement through mycelium and the frequency with which compatible *C. parasitica* strains undergo anastomosis, it is quite conceivable that an extracellular route of infection and packaging functions became dispensable once the ancestral virus encountered the environment of a fungal host. Similarly, the absence of a requirement for packaging of a genomic ssRNA could have resulted in an altered ratio of genomic to replicative form dsRNA, leading to a situation in which a dsRNA form became predominant. A functional parallel can also be drawn between the separation of ORFA and ORFB within L-dsRNA by introduction of a pentanucleotide (5'-UAAUG-3'), with the potential for translation termination and reinitiation, and the apparent separation of similar coding domains into individual RNA segments in the case of BaYMV. Although the relative order of the helicase and polymerase domains found in the potyviruses and BaYMV is typical for positive-strand RNA viruses, the reverse order observed in L-dsRNA has also been reported for coronaviruses and totoviruses (17, 49, 96). In considering the likelihood of an evolutionary transposition event involving the two domains as proposed in step (ii), one is reminded of the frequency with which L-dsRNA appears to undergo internal deletion

events and the recent evidence suggesting recombination events involving L-dsRNA and cellular RNAs (92).

The possible common origin of a fungal viruslike genetic element and the plant potyviruses also raises the question of whether the host of the postulated ancestral virus was a plant or a fungus. BaYMV is in fact transmitted in nature by a fungal vector (73), although it is unclear whether BaYMV has the ability to replicate in the vector. Since fungi are known to interact with plants at many different levels in nature, one could imagine a series of events which could lead to the acquisition of a potylike virus by a fungus during saprophytic or pathogenic interactions with a plant host. In this context, Koonin et al. (63) suggested that since L-dsRNA reduces the virulence of *C. parasitica*, acquisition of an ancestral virus may have been advantageous by increasing the survival of the plant host and, consequently, the fungal pathogen. Nearly all mycoviruses have genomes composed of dsRNA (19, 68). Thus an alternative, although less plausible, possibility is that an ancestral dsRNA was transmitted from a fungal host to a plant and that this was followed by evolution to an ssRNA virus along the lines recently suggested by Bruenn (18). Although these evolutionary considerations are clearly speculative, further comparisons of L-dsRNA- and potyvirus-encoded amino acid sequence information are likely to have practical benefits, possibly leading to identification of additional functional domains such as other proteinases or VPg-like sequences (95).

VIRUS-MEDIATED MODULATION OF *C. PARASITICA* GENE EXPRESSION

In addition to reduced levels of virulence, *C. parasitica* strains harboring viruslike dsRNAs often exhibit other properties, termed hypovirulence-associated traits, that distinguish them from corresponding isogenic dsRNA-free virulent strains. These can include altered colony morphology (3, 6, 42), reduced levels of certain enzymatic activities such as laccase (88), reduced levels of oxalate accumulation (56), and suppressed conidiation (asexual sporulation) (6, 41). However, different hypovirulent strains exhibit a range of associated traits, and the phenotype exhibited by a particular strain appears to be a function of the viral genetic element rather than the fungal genotype (42). In addition, Hillman et al. (58) reported that the suppression of fungal processes observed in hypovirulent strains can be partially relieved by exposure to high light intensity, suggesting the possibility that both light and HAV dsRNAs may influence fungal gene expression through the same or converging regulatory pathways. In spite of these observations, there remains some debate about whether hypovirulence is related to a general debilitation of fungal metabolism or is the consequence of alterations in the expression of specific fungal genes and regulatory pathways. A related question is whether hypovirulence and associated traits are the result of a general reaction of the fungal host to the presence of replicating viral RNAs or are a response to the action of specific viral coding domains. The identification and cloning of fungal genes that appear to be targets of viral modulation and the development of methods for stably transforming virulent *C. parasitica* strains with cDNA copies of L-dsRNA coding domains have begun to clarify these issues.

Evidence for Virus-Mediated Modulation of Specific Fungal Genes and Regulatory Pathways

Two reports by Powell and Van Alfen (84, 85) provided the first direct indication that specific fungal genes are expressed differentially in virulent and hypovirulent *C. parasitica* strains. In the first report, the authors used differential hybridization analysis to identify several RNA species that accumulated to a high concentration in virulent strains but were undetectable in RNA samples prepared from isogenic hypovirulent strains. At least one additional RNA species that appeared to preferentially accumulate in hypovirulent strains was identified. In the second report, the accumulation of several fungal proteins was shown by two-dimensional gel electrophoretic analysis to be selectively reduced in hypovirulent strains. The nature of the polypeptides or the coding potential of the RNA species identified in those studies have not yet been reported.

Several subsequent studies have reported hypovirulence-associated down-regulation of proteins with known enzymatic activities or structural properties. Rigling et al. (88) reported that the phenol oxidase activity of the laccase type was significantly reduced in hypovirulent *C. parasitica* strains compared with the level observed in isogenic virulent strains. This result was confirmed for the paired isogenic virulent and hypovirulent strains EP155 and EP713 by Hillman et al. (58). More recently, Carpenter et al. (21) reported a similar, although less substantial, down-regulation of a developmentally regulated tissue-specific cell surface protein called cryparin in hypovirulent *C. parasitica* strains.

Although correlative studies measuring the relative levels of enzymatic activity or amount of structural proteins present in virulent and hypovirulent strains are necessary and informative, a detailed understanding of the underlying mechanisms involved in HAV-mediated modulation of fungal gene expression requires the molecular characterization of specific fungal genes that serve as targets of such modulation. The laccase gene provided an attractive candidate for such studies for several reasons. Its activity was shown to be down-regulated in a number of different hypovirulent strains (88), including strain EP713 with its well characterized viral dsRNA (58). In addition, laccase activity has been detected in many fungal species and has been implicated as playing a role in fungal sporulation (67), pigmentation (31, 69), lignin degradation (14, 62), and pathogenesis (48, 72).

Rigling and Van Alfen (89) were able to obtain a partial cDNA clone of the *C. parasitica* laccase gene by screening a cDNA expression library with monospecific antisera prepared against purified laccase. This allowed these investigators to monitor the relative accumulation of laccase mRNA in hypovirulent and virulent strains by Northern (RNA) analysis. Under the culture and inoculation conditions used, laccase activity and laccase mRNA accumulation were shown to be reduced by 75 and 85%, respectively, in the hypovirulent strain (strain UEP1, derived from the same European hypovirulent strain as was EP713 [84]) relative to the levels found in the isogenic virulent strain (EP155/2 [84]), whereas there was no significant difference in the level of biomass produced by the two cultures. These results indicated that down-regulation of laccase synthesis in hypovirulent strains occurs at the level of laccase mRNA transcription or stability.

Independently, Choi et al. (24) reported the cloning and extensive characterization of the entire *C. parasitica* laccase gene. These investigators indicated that laccase gene expression was under complex regulatory control and that the level
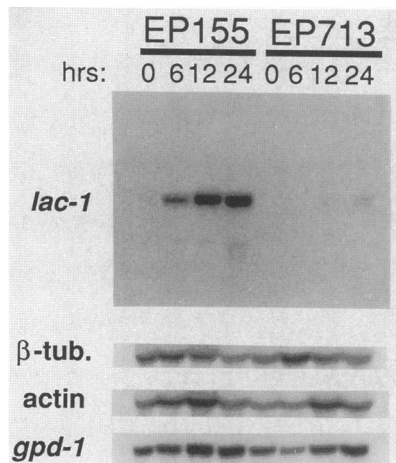
FIG. 8. Northern hybridization analysis of EP155 and EP713 RNAs after addition of cycloheximide. RNA prepared from strains EP155 and EP713 at various times after the addition of 3 μM cycloheximide were separated through a formaldehyde–1.5% agarose gel, transferred to a nylon membrane, and hybridized sequentially with probes specific for laccase (*lac-1*), β-tubulin, actin, and glyceraldehyde-3-phosphate dehydrogenase (*gpd-1*). Estimated sizes of the different mRNAs were as follows: *lac-1*, 2.3 kb; β-tubulin, 1.9 kb; actin, 1.8 kb; *gpd-1*, 1.5 kb. The times after addition of cycloheximide at which the RNA samples were harvested are indicated at the top of the autoradiogram. Reprinted with permission from *Molecular Plant-Microbe Interactions* (24).

of laccase mRNA accumulation appeared to be influenced by a variety of conditions including the culture medium, the age of the culture, and exposure to light. They further indicated that although laccase mRNA accumulation was generally found to be substantially lower in hypovirulent strain EP713 than in the isogenic virulent strain EP155, quantification was complicated by the variability observed within one set and between different sets of conditions. In an effort to reduce the problem of variability, a protocol previously reported to consistently induce laccase activity in *Neurospora crassa*, treatment with the protein synthesis inhibitor cycloheximide (43), was tested on cultures of *C. parasitica*. As shown by the Northern hybridization analysis in Fig. 8, addition of 3 μM cycloheximide to cultures of the virulent strain EP155 resulted in a time-dependent increase in the accumulation of laccase mRNA, reaching a maximum stimulation of approximately 15- to 20-fold by 24 h. Rehybridization of the same blot with probes specific for mRNAs that encode two cellular structural proteins, β-tubulin and actin, and an mRNA encoding the highly expressed protein glyceraldehyde-3-phosphate dehydrogenase (*gpd-1*) (25) revealed little change in accumulation of these mRNAs over the 24-h incubation period. The differential effect on the accumulation of the different mRNAs clearly indicated that the cycloheximide-mediated induction of *C. parasitica* laccase mRNA levels is under the control of a selective regulatory pathway. Significantly, treatment of the isogenic hypovirulent strain EP713 with cycloheximide resulted in only a very modest increase in laccase mRNA accumulation, whereas the accumulation of β-tubulin, actin, and *gpd-1* mRNAs was essentially unaffected either by the presence of viral dsRNA or by cycloheximide addition. Although limited to only a few fungal genes, these results strongly support the proposal that the hypovirulence phenotype is a consequence of altered expression of a specific subset of fungal genes rather than a general debilitation of the fungus.

The identification and cloning of a target gene for HAV dsRNA-mediated down-regulation provides new opportunities for examining the regulatory pathways involved in the manifestation of hypovirulence. For example, Choi et al. (24) have discussed the possibility that the cycloheximide-mediated increase in laccase mRNA accumulation observed for strain EP155 represents a derepression of laccase gene expression. By using a gene reporter system driven by the laccase promoter region, it should now be possible to identify the DNA control elements involved in laccase derepression. Since derepression is prevented in the presence of HAV dsRNA, this information is likely to lead to a clearer understanding of the mechanisms involved in virus-mediated modulation of fungal gene expression. Consequently, it is anticipated that this system will offer unique opportunities for future detailed studies of signal transduction pathways in a plant-pathogenic fungus.

Contribution of Individual Viral Coding Domains to the Hypovirulence Phenotype

The recent progress in cloning and characterization of a hypovirulence-associated viral dsRNA described above and the development of a DNA-mediated transformation system for *C. parasitica* (30) have provided the opportunity to ask directly whether the phenotypic traits exhibited by hypovirulent strains are the result of a general response of the fungal host to the physical presence of replicating viral dsRNA or a consequence of the action of specific virus-encoded gene products. The potential phenotypic contribution of the 5'-proximal coding domain, ORFA, of L-dsRNA found in strain EP713 was tested by Choi and Nuss (26), who transformed virulent strain EP155 with plasmid pAXHY2 (described in Fig. 9). This plasmid construct contained a cDNA copy of ORFA fused upstream to the *C. parasitica gpd-1* promoter and fused downstream to the *gpd-1* terminator (25). It also contained the *Escherichia coli* hygromycin B phosphotransferase gene as a selectable marker flanked by the *trpC* promoter and terminator from *Aspergillus nidulans* (33). A plasmid that contained ORFA in the reverse orientation, pAXHY5, served as a control. These transformation studies were aided by the convenient phenotypic markers exhibited by the isogenic virulent and hypovirulent strains EP155 and EP713, respectively. Under standard laboratory conditions, virulent strain EP155 produces orange pigments, conidiates abundantly, and produces easily measurable levels of laccase activity. In contrast, hypovirulent strain EP713, which differs genetically from EP155 only by the presence of viral dsRNAs, is white, is suppressed in conidiation, and produces very reduced levels of laccase activity (58). The growth rate on agar medium is also slightly lower for strain EP713 than for strain EP155 (58).

Transformation with the antisense (−) ORFA plasmid pAXHY5 resulted in no alteration in morphology or reduction in pigmentation, indicating that vector sequences did not visibly alter fungal phenotype (Fig. 10). In contrast, most hygromycin B-resistant colonies resulting from transformation with the sense (+) ORFA construct pAXHY2 resembled strain EP713 rather than the untransformed strain EP155. In addition to an obvious reduction in the production of orange pigment, the (+) ORFA transformants also produced 16- to 40-fold fewer conidia than the (−) ORFA transformants or untransformed strain EP155 and were significantly reduced in laccase production. The combined results provided the
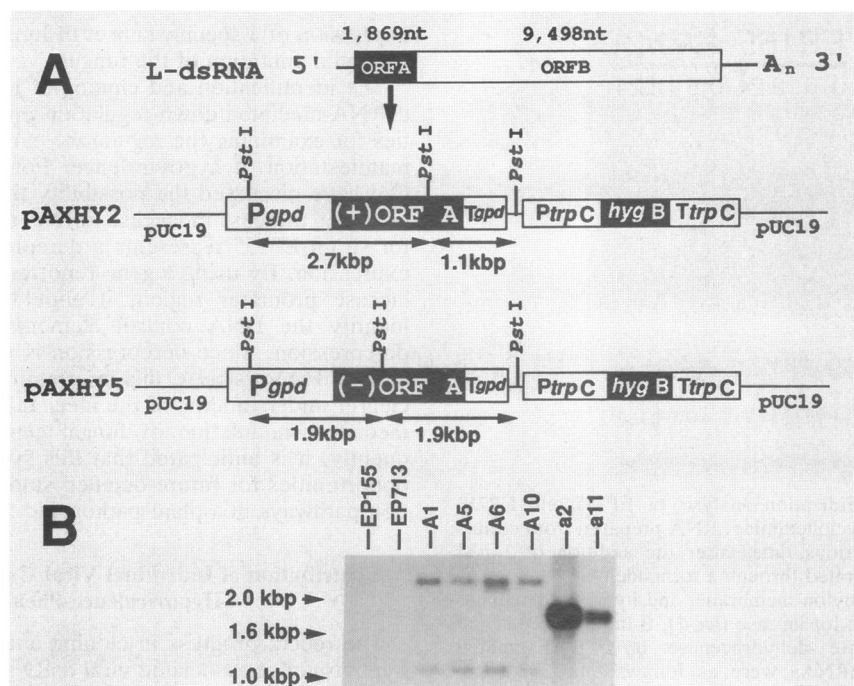
FIG. 9. Description of transformation plasmids pAXHY2 and pAXHY5 and Southern analysis of *C. parasitica* transformants. (A) Plasmid pAXHY2 contains the first coding domain of the EP713 L-dsRNA, ORFA, flanked by the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase gene promoter (P*gpd*) and terminator (T*gpd*). The *gpd-1* promoter region extended 1,696 nt upstream of map position 376 of the published *gpd-1* sequence, and the terminator extended from map positions 1571 to 2172 (25). This plasmid also contains the *E. coli* hygromycin B phosphotransferase gene (*hyg*B) preceded by the *Aspergillus nidulans trpC* promoter (P*trpC*) (33), all in a pUC19 background. Plasmid pAXHY5 is the same as pAXHY2 except that the ORFA coding domain is in the antisense orientation. The locations of *Pst*I sites within and flanking the ORFA coding region are also indicated. The arrows located under the ORFA region in each plasmid indicate the sizes of ORFA-containing fragments that are expected to be liberated by *Pst*I digestion. (B) Southern analysis of *Pst*I-digested genomic DNA prepared from four (+) ORFA transformants (A1, A5, A6, and A10), two (−) ORFA transformants (a2 and a11), untransformed strain EP155, and hypovirulent strain EP713, all probed with the ORFA coding sequence. The positions of marker fragments are indicated at the left. Reprinted with permission from *EMBO Journal* (26).

first direct evidence for a cause-and-effect relationship between the viral dsRNA present in a hypovirulent *C. parasitica* strain and specific traits associated with that strain. In addition, they demonstrated that these phenotypic traits are not the result of some general reaction of the fungus to replicating viral RNA but are caused by the action of specific virus-encoded gene products.

Interestingly, when inoculated onto dormant chestnut stems to test virulence levels, (+) ORFA transformants produced cankers of approximately the same size as those produced by the (−) ORFA transformants and strain EP155. In other words, on the basis of this assay, there was no evidence that the (+) ORFA transformants were reduced in virulence. The apparent uncoupling of hypovirulence-associated traits, such as suppressed sporulation, from hypovirulence in the (+) ORFA transformants could be related to one of several factors. The complete hypovirulence phenotype may require a higher level of ORFA expression or the contribution of additional virus-encoded gene products. Alternatively, the genetic information responsible for hypovirulence may lie outside ORFA entirely. Although intrinsically interesting, studies aimed at determining the contribution of individual virus-encoded polypeptides to the hypovirulence phenotype have taken on a more practical aspect with the development of an infectious cDNA clone of L-dsRNA, as described in the following section.

ENGINEERED TRANSMISSIBLE HYPOVIRULENCE

In the event that transformation studies such as those described above were to identify successfully a specific viral gene product that was responsible for virulence attenuation, practical application of that information for the engineering of more effective hypovirulent strains would be limited for several reasons. The fact that hypovirulence-associated viral genetic information is in the form of RNA rather than DNA precludes easy genetic manipulation. Furthermore, these viral genetic elements are not infectious in the classical sense and have never been reintroduced into fungal strains in a form that was subsequently able to replicate. Fortunately, the recent development of an infectious full-length cDNA clone of L-dsRNA (27) represents a major advancement in overcoming these limitations.

The possibility that these genetic elements would be amenable to manipulation for the purpose of genetically engineering more effective hypovirulent *C. parasitica* strains was, in fact, one of the main considerations in initiating the molecular characterization of hypovirulence-associated viruses. The successful cloning and sequence determination of L-dsRNA presented several basic options for accomplishing this goal. One option involved the generation of a full-length synthetic transcript corresponding to the L-dsRNA coding strand that could be introduced into fungal spheroplasts by transfection. Once internalized, the viral transcripts would
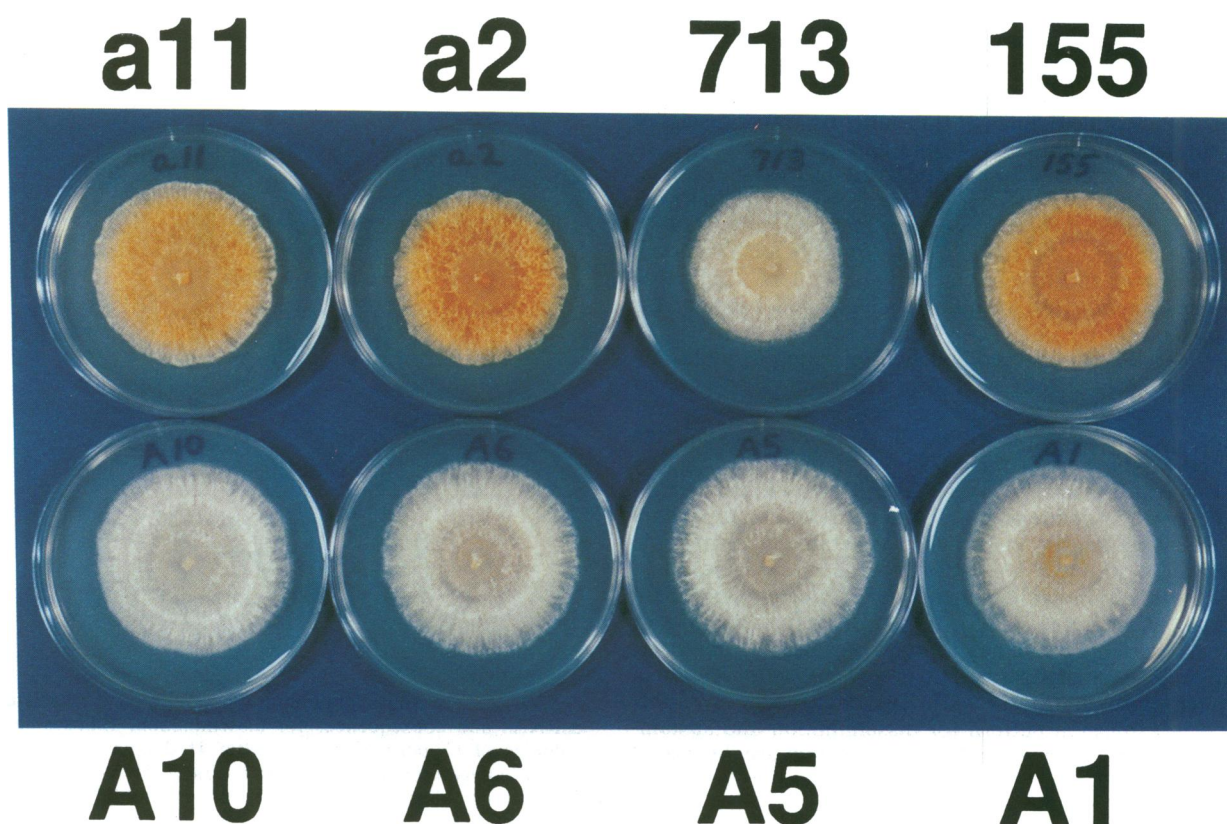
FIG. 10. Effect of ORFA transformation on pigmentation and colony morphology. Transformation of EP155 spheroplasts was performed essentially as described previously (30), followed by selection on medium containing hygromycin B (40 mg/ml). Transformants used in this study were further selected on a higher concentration of hygromycin B (200 mg/ml). Transformants containing either plasmid pAXHY2 [(+) ORFA transformants A1, A5, A6, or A10] or plasmid pAXHY5 [(−) ORFA transformants a2 and a11] were grown in parallel with untransformed EP155 and the isogenic hypovirulent strain EP713 for 5 days on potato dextrose agar (Difco) on the laboratory bench with a light intensity of <2,000 lx and at a temperature of 22 to 24°C as described previously (58). Reprinted with permission from *EMBO Journal* (26).

presumably serve as mRNA for production of virus-encoded replicative proteins and as template for RNA-dependent replication, as was reported initially for brome mosaic virus in barley protoplasts (1). A second option, the one adopted by Choi and Nuss (27), involved direct transformation with a plasmid vector containing the complete cDNA copy of L-dsRNA, as previously demonstrated for poliovirus (86) and bacteriophage Qβ (97). Success of this strategy requires the generation of transcripts corresponding to the L-dsRNA coding strand directly from chromosomally integrated cDNA copies of L-dsRNA under the control of promoter and terminator elements supplied on the transformation vector. The advantage that this option provides for purposes of engineering hypovirulent *C. parasitica* strains is that once integrated into the fungal chromosome, the viral cDNA copy would have the potential for stable transmission through both asexual and sexual spores, a point that will be expanded upon later in this section.

The transformation vector used to deliver the full-length cDNA copy of L-dsRNA was a derivative of plasmid pAXHY2 (Fig. 9) in which the ORFA coding region was replaced with the complete L-dsRNA sequence. As was observed previously for the (+) ORFA transformants (26), most of the hygromycin-resistant colonies resulting from transformation of strain EP155 with the complete cDNA copy of L-dsRNA exhibited hypovirulence-associated traits such as reduced pigmentation, suppressed sporulation, and

reduced laccase production. However, these transformed strains differed from the (+) ORFA transformants in several fundamental properties. All transformants that exhibited hypovirulence-associated traits were also found to harbor a cytoplasmically replicating form of L-dsRNA that was shown with the aid of a molecular tag to be directly resurrected from the integrated cDNA copy. Moreover, the resurrected viral RNA was shown to convert compatible virulent strains to exhibit hypovirulence-associated following anastomosis. Finally, both the original transformants and strains converted by the resurrected L-dsRNA were shown to be hypovirulent. Thus, unlike the (+) ORFA transformants that exhibited hypovirulence-associated traits but were not reduced in virulence, transformants containing the full-length cDNA copy of L-dsRNA exhibited the complete transmissible hypovirulence phenotype.

The results reported by Choi and Nuss (27) constitute direct evidence that a viruslike dsRNA is the genetic determinant responsible for transmissible hypovirulence in the chestnut blight fungus. The introduction of a biologically active cDNA intermediate to create an artificial infectious replication cycle for an HAV dsRNA also has significant experimental and practical implications. It is now possible to precisely alter the genetic makeup of L-dsRNA by site-directed mutagenesis of its cDNA copy. Collections of defined L-dsRNA mutants can now readily be generated for examining questions related to L-dsRNA gene expression

(e.g., proteolytic processing or translational control at the ORFA-ORFB junction) and replication (e.g., the roles of the putative RNA-dependent RNA polymerase, helicase, and terminal nucleotide domains). It is anticipated that results of such fundamental studies will find application in efforts to engineer more effective hypovirulent strains. As an example, since ORFA has been shown to suppress sporulation (26), one could imagine that by appropriately mutating ORFA within the context of the infectious L-dsRNA cDNA clone, it may be possible to produce a hypovirulent strain that sporulates abundantly, resulting in increased capacity for dissemination.

The fact that an autonomously replicating cytoplasmic RNA form of L-dsRNA is resurrected from cDNA copies integrated into the fungal genome has interesting implications for current efforts to restore the American chestnut. As repeatedly indicated in this review and illustrated in Fig. 11A, efficient transmission of HAV dsRNAs and the accompanying hypovirulence phenotype to a virulent strain occurs only following anastomosis with a vegetatively compatible hypovirulent strain. HAV dsRNAs appear not to be transmitted to ascospores following mating between hypovirulent and virulent strains (5, 6, 45). Intrastrain transmission does occur at a variable frequency through conidia (91); however, the overall level of conidiation is severely suppressed in many hypovirulent strains (42). The consequences of this natural transmission pattern for dissemination and sustainability of hypovirulence in a forest ecosystem are illustrated in simplified form in Fig. 11B. Introduction of a natural hypovirulent C. parasitica strain results in the efficient conversion only of virulent strains that are members of the same or closely related vegetative compatibility groups. This could result in effective disease control if the compatible strains represent a significant portion of the total population. If, however, the vegetative compatibility structure of the population is complex, as appears to be the case in the eastern deciduous forest of the United States (13), the proportion of virulent strains that would be susceptible to conversion would be small, thus limiting vegetative spread and the effectiveness of disease control. Field observations are in general agreement with this model (3, 11, 13, 71).

The transmission pattern predicted for engineered hypovirulent strains containing integrated cDNA copies of L-dsRNA differs substantially from that observed for natural hypovirulent strains, as illustrated in Fig. 12A. The primary mode of transmission for the resurrected form of L-dsRNA would remain via anastomosis with compatible strains. Significantly, the integrated cDNA form would have the potential of being transmitted to ascospores resulting from sexual crosses. Although natural hypovirulent strains do not serve as female partners in laboratory mating, they can readily serve as male partners (8). Nuclear inheritance of the integrated cDNA would be followed by resurrection of the cytoplasmic L-dsRNA form in the progeny, a portion of which will represent vegetative compatibility groups different from the parental strains as a result of allelic rearrangement. Additionally, nearly all, rather than a fraction, of the asexual spores would be expected to contain L-dsRNA genetic information either in a cDNA form from which the L-dsRNA form can be resurrected or in the combined cDNA and dsRNA forms.

As illustrated in Fig. 12B, the transmission properties of such engineered strains should result in increased dissemination and sustainability of the hypovirulence phenotype. Unlike the vegetative compatibility system in C. parasitica,
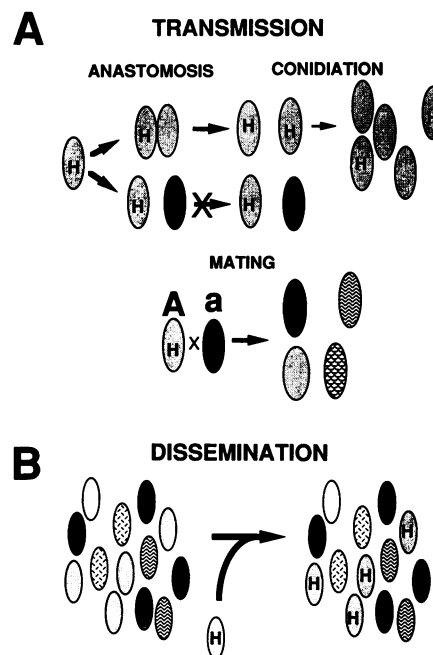

FIG. 11. Transmission of natural hypovirulence-associated viral dsRNAs and consequences for dissemination. Ellipses represent colonies of C. parasitica. The letter H denotes cytoplasmically replicating hypovirulence-associated viral dsRNAs. Different shading patterns indicate different vegetative compatibility groups. That is, colonies that are in the same vegetative compatibility group are highlighted with the same shading pattern. (A) The three potential modes of transmission of the viral dsRNA. Efficient transmission of viral dsRNA to a virulent strain occurs only following anastomosis (fusion of hyphae). However, anastomosis is limited to strains of the same or very closely related vegetative compatibility groups. Consequently, viral dsRNAs are not transmitted to incompatible strains (indicated by X through arrow). A second potential mode of transmission is through asexual spores (conidia). Transmission to conidia occurs at a variable frequency. A third potential mode of transmission is through sexual spores (ascospores) resulting from mating. Mating compatibility in C. parasitica is controlled by a single mating-type locus involving two alleles designated (A) and (a) (5). Owing to allelic rearrangement, the progeny of a sexual cross can be of different vegetative compatibility groups from the parental strains. However, available evidence indicates that hypovirulence-associated viral dsRNAs are not transmitted through ascospores at readily detectable levels. (B) Predicted consequences of this transmission pattern on the dissemination of introduced natural hypovirulence. Introduction of a natural hypovirulent C. parasitica strain into a forest ecosystem results in efficient conversion only of virulent strains that are of the same or closely related vegetative compatibility groups.

sexual compatibility is controlled by a single mating-type locus involving two alleles designated "A" and "a" (5) and would be expected to pose few barriers to the spread of the integrated L-dsRNA cDNA copy through the population by sexual crossing. Thus, the integrated L-dsRNA genetic information could be transmitted to progeny of a vegetatively incompatible strain by mating. Resurrection of the cytoplasmic L-dsRNA form would result in subsequent vegetative dissemination via anastomosis through all vegetative compatibility groups represented in the progeny population. Moreover, a higher proportion of the asexual spores derived from the introduced and converted hypovirulent
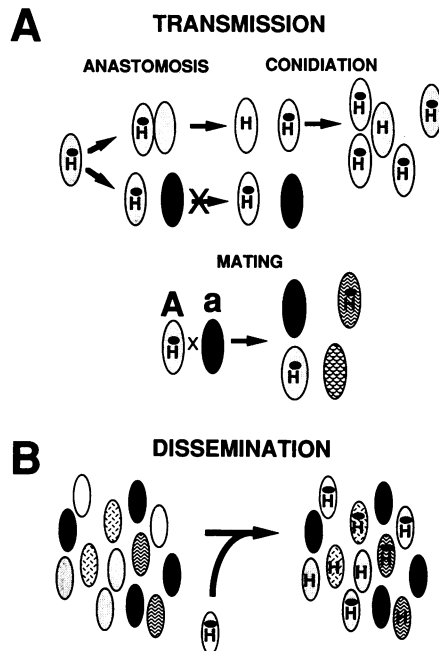
A TRANSMISSION

FIG. 12. Predicted transmission and dissemination of genetically engineered hypovirulent *C. parasitica* strains. Designations are the same as in Fig. 11, except that the integrated cDNA copy of a hypovirulence-associated dsRNA is indicated by the solid black oval within colonies. (A) The predicted transmission pattern for a genetically engineered hypovirulent strain differs from that of a natural hypovirulent strain in two fundamental ways. First, nearly all, rather than a small fraction, of the asexual spores (conidia) would be expected to carry L-dsRNA genetic information either in the form of integrated cDNA from which L-dsRNA can be resurrected or in the combined forms of integrated cDNA and resurrected dsRNA. Second, and more significantly for purposes of dissemination, the integrated cDNA form of L-dsRNA has the potential for transmission into the progeny of sexual crosses. This would result in the introduction of the integrated cDNA and resurrected forms of L-dsRNA into strains representing vegetative compatibility groups other than that of the parental hypovirulent strain. (B) The predicted ability of the cDNA form of L-dsRNA to be introduced into new vegetative compatibility groups as a result of mating coupled with a higher transmission frequency through asexual spores is likely to result in significantly increased dissemination and sustainability of the hypovirulence phenotype after introduction of a genetically engineered *C. parasitica* strain.

strains would contain L-dsRNA genetic information and would thus be able to initiate hypovirulent colonies. Additional increases in the rate of dissemination could be forthcoming as a result of genetic manipulation of L-dsRNA at the cDNA level, e.g., development of abundantly conidiating hypovirulent strains. Since the surviving root systems of blight-infested American chestnut trees continue to produce sprouts throughout its natural range (3, 71), it is conceivable that the release of improved genetically engineered hypovirulent strains could significantly contribute to the restoration of this once important forest species. The feasibility of this approach would, of course, be contingent upon a thorough assessment of issues such as potential alterations in host range of the engineered strains, ecological fitness, meiotic stability of integrated L-dsRNA cDNA copies, and mitotic stability of the resurrected L-dsRNA.

CONCLUSIONS AND FUTURE PROSPECTS

The eventual outcome of any host-pathogen interaction is ultimately determined by a complex series of counteracting responses. Initial interactions between a potential pathogen and a prospective host often trigger pathogenic responses required to breach physical defense barriers or to initiate colonization at wound sites. The prospective host responds by elaborating an array of defense mechanisms. The pathogen counters with measures designed to inactivate or evade host defense responses. Recent application of molecular analysis has revealed a myriad of plant defense responses to microbial attack. In several instances, these responses have been shown to involve transcriptional activation of specific genes and the induction of regulatory cascades that result in a significant change in the pattern of host gene expression (for recent reviews, see references 16, 36, 37, 50, and 66). Although less well studied, pathogenic responses must also involve the expression of different sets of genes required to initiate and establish infections and to inactivate or evade defense responses. In this regard, one of the attractions for studying transmissible hypovirulence in *C. parasitica* was the anticipation that by understanding how a resident viral genetic element was able to attenuate virulence, it might be possible to gain insight into the nature and regulation of pathogenic responses. Recent progress in developing the molecular biology of this system has reinforced this view.

Elucidation of the genetic organization and basic expression strategy of a hypovirulence-associated viral RNA, the availability of cDNA clones representing the entire viral RNA, and the development of a DNA-mediated transformation system have provided the means for determining whether individual viral gene products contribute directly to the hypovirulence phenotype. Efforts invested in the cloning and detailed characterization of *C. parasitica* nuclear genes have facilitated the development of transformation vectors by providing homologous transcription regulatory elements and have generated reagents essential for examining the influence of virus on nuclear gene expression. These approaches have already provided strong evidence that individual viral gene products selectively modulate the expression of specific subsets of fungal genes by perturbing specific regulatory pathways. The recent development of an infectious cDNA clone of a hypovirulence-associated viral RNA will certainly expedite efforts to identify the precise viral genetic information responsible for hypovirulence as well as fungal host factors that mediated the response. A mechanistic understanding of transmissible hypovirulence will undoubtedly lead to a clearer indication of the determinants that contribute to fungal virulence. As a result of these recent developments, *C. parasitica* now provides an attractive experimental system for examining signal transduction pathways in the context of host-pathogen interactions from the perspective of the pathogen.

Efforts to assess the practical implications related to the newly acquired ability to genetically engineer hypovirulent *C. parasitica* strains will require a multiphased approach. Laboratory studies have already determined that integrated cDNA copies of L-dsRNA are mitotically stable (23a), and studies designed to examine meiotic stability are in progress. The second phase will require testing of parameters such as host range, fitness, and dissemination under simulated field conditions in appropriate containment facilities to satisfy current guidelines for the environmental release of genetically engineered microorganisms. The third and most exciting phase will involve controlled field studies to determine

the efficacy of the engineered strains for control of chestnut blight. The broad scope of these studies will necessitate a multidisciplinary approach involving input from plant pathologists, ecologists, foresters, mycologists, and molecular biologists.

The availability of an infectious cDNA clone of a hypovirulence-associated viral RNA also provides opportunities for determining the feasibility of expanding this form of biological control to other pathogenic fungi. Studies are currently in progress to determine whether fungal species other than *C. parasitica* can support the replication of L-dsRNA and the resulting phenotypic consequences. Possible complications include host range restrictions with respect to both L-dsRNA replication and the manifestation of hypovirulence. Replication and expression of L-dsRNA may be dependent on specific *C. parasitica*-encoded factors. The mechanism underlying hypovirulence may require highly specific, evolutionarily defined protein-protein or protein-nucleic acid interactions involving viral and host factors. In this regard, virulence-modulating viruses and unencapsidated dsRNAs have been reported for a large number of plant-pathogenic fungi (34, 78). These observations suggest the possibility that the same concept of virus-mediated biocontrol envisioned for genetically engineered strains of *C. parasitica* could be applied to other pathogenic fungi by using endogenous hypovirulence agents.

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REFERENCES

1. **Ahlquist, P., R. French, M. Janda, and L. S. Loesch-Fries.** 1984. Multicomponent RNA plant infection derived from cloned viral cDNA. Proc. Natl. Acad. Sci. USA **18:**7066–7070.
2. **Anagnostakis, S. L.** 1977. Vegetative incompatibility in *Endothia parasitica*. Exp. Mycol. **1:**306–316.
3. **Anagnostakis, S. L.** 1982. Biological control of chestnut blight. Science **215:**466–471.
4. **Anagnostakis, S. L.** 1982. Genetic analysis of *Endothia parasitica*: linkage data for four single genes and three vegetative compatibility types. Genetics **102:**25–28.
5. **Anagnostakis, S. L.** 1984. The mycelial biology of *Endothia parasitica*. II. Vegetative incompatibility, p. 499–507. *In* D. H. Jennings and A. D. M. Rayner (ed.), The ecology and physiology of the fungal mycelium. Cambridge University Press, Cambridge.
6. **Anagnostakis, S. L.** 1984. Nuclear gene mutations in *Endothia* (*Cryphonectria*) *parasitica* that affect morphology and virulence. Phytopathology **74:**561–565.
7. **Anagnostakis, S. L.** 1987. Chestnut blight: the classical problem of an introduced pathogen. Mycologica **79:**23–37.
8. **Anagnostakis, S. L.** 1988. *Cryphonectria parasitica*, cause of chestnut blight, p. 123–136. *In* G. S. Sidhu (ed.), Advances in plant pathology. Academic Press, Ltd., London.
9. **Anagnostakis, S. L.** 1990. Improved chestnut tree condition maintained in two Connecticut plots after treatments with hypovirulent strains of the chestnut blight fungus. Forest Sci. **36:**113–124.
10. **Anagnostakis, S. L., and P. R. Day.** 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology **69:**1226–1229.
11. **Anagnostakis, S. L., B. Hau, and J. Kranz.** 1986. Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in

Connecticut and Europe. Plant Dis. **70:**536–538.
12. **Anagnostakis, S. L., and R. A. Jaynes.** 1973. Chestnut blight control: use of hypovirulent cultures. Plant Dis. Rep. **57:**225–226.
13. **Anagnostakis, S. L., and J. Kranz.** 1987. Population dynamics of *Cryphonectria parasitica* in a mixed-hardwood forest in Connecticut. Phytopathology **77:**751–754.
14. **Ander, P., and K.-E. Eriksson.** 1976. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentrum*. Arch. Microbiol. **109:**1–8.
15. **Biraghi, A.** 1953. Possible active resistance to *Endothia parasitica* in *Castanea sativa*, p. 643–645. *In* Reports to 11th Congress of the International Union of Forest Research Organizations. International Union of Forest Research Organizations, Rome.
16. **Bowles, D. J.** 1990. Defense-related proteins in higher plants. Annu. Rev. Biochem. **59:**873–907.
17. **Bredenbeck, P. J., C. J. Pachuk, A. F. H. Noten, J. Charite, W. Luytjes, S. R. Weiss, and W. J. M. Spaan.** 1990. The primary structure and expression of the second open reading frame of the polymerase gene of the coronavirus MHV-59: a highly conserved polymerase is expressed by an efficient ribosomal frameshift mechanism. Nucleic Acids Res. **18:**1825–1832.
18. **Bruenn, J. A.** 1991. Relationship among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerase. Nucleic Acids Res. **19:**217–226.
19. **Buck, K. W.** 1986. Fungal virology—an overview, p. 2–84. *In* K. W. Buck (ed.), Fungal virology. CRC Press, Boca Raton, Fla.
20. **Burnham, C. R.** 1988. The restoration of the American chestnut. Am. Sci. **76:**478–487.
21. **Carpenter, C. E., R. J. Mueller, P. Kazmierczak, L. Zhang, D. K. Villalon, and N. K. van Alfen.** 1992. Effect of a virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria* (*Edothia*) *parasitica*. Mol. Plant Microbe Interact. **4:**55–61.
22. **Carrington, J. C., S. M. Cary, T. D. Parks, and W. G. Dougherty.** 1989. A second proteinase encoded by a plant potyviral genome. EMBO J. **8:**365–370.
23. **Carrington, J. C., and K. L. Herndon.** 1992. Characterization of the potyviral HC-Pro autoproteolytic cleavage site. Virology **187:**308–315.
23a.**Chen, B., G. H. Choi, and D. L. Nuss.** Unpublished data.
24. **Choi, G. H., T. G. Larson, and D. L. Nuss.** 1992. Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. Mol. Plant Microbe Interact. **5:**119–128.
25. **Choi, G. H., and D. L. Nuss.** 1990. Nucleotide sequence of the glyceraldehyde-3-phosphate dehydrogenase gene from *Cryphonectria parasitica*. Nucleic Acids Res. **18:**5566.
26. **Choi, G. H., and D. L. Nuss.** 1992. A viral gene confers hypovirulence-associated traits to the chestnut blight fungus. EMBO J. **11:**473–477.
27. **Choi, G. H., and D. L. Nuss.** Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science **257:**800–803.
28. **Choi, G. H., D. M. Pawlyk, and D. L. Nuss.** 1991. The autocatalytic protease p29 encoded by a hypovirulence-associated virus of the chestnut blight fungus resembles the potyvirus-encoded protease HC-Pro. Virology **183:**747–752.
29. **Choi, G. H., R. Shapira, and D. L. Nuss.** 1991. Co-translational autoproteolysis involved in gene expression from a double-stranded RNA genetic element associated with hypovirulence of the chestnut blight fungus. Proc. Natl. Acad. Sci. USA **88:**1167–1171.
30. **Churchill, A. C. L., L. M. Ciuffetti, D. R. Hansen, H. D. van Etten, and N. K. van Alfen.** 1990. Transformation of the fungal pathogen *Cryphonectria parasitica* with a variety of heterologous plasmids. Curr. Genet. **17:**25–31.
31. **Clutterbuck, A. J.** 1972. Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J. Gen. Microbiol. **70:**423–435.
32. **Cochran, M. F.** 1990. Back from the brink: chestnuts. Natl.

Geogr. Mag. **177:**128–140.

32a. Craven, M., and D. L. Nuss. Unpublished observations.

33. Cullen, D., S. A. Leong, L. J. Wilson, and D. J. Henner. 1987. Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. Gene **57:**21–26.

34. Day, P. R., and J. A. Dodds. 1979. Viruses of plant pathogenic fungi, p. 201–238. *In* P. A. Lemke (ed.), Viruses and plasmids in fungi. Marcel Dekker Inc., New York.

35. Day, P. R., J. A. Dodds, J. E. Elliston, R. A. Jaynes, and S. L. Anagnostakis. 1977. Double-stranded RNA in *Endothia parasitica*. Phytopathology **67:**1393–1396.

36. Dixon, R. A., and M. P. Harrison. 1990. Activation, structure, and organization of genes involved in microbial defense in plants. Adv. Genet. **28:**165–234.

37. Dixon, R. A., and C. J. Lamb. 1990. Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41:**339–367.

38. Dodds, J. A. 1980. Association of type 1 viral-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica*. Virology **107:**1–12.

39. Dodds, J. A. 1980. Revised estimates of the molecular weights of dsRNA segments in hypovirulent strains of *Endothia parasitica*. Phytophathology **70:**1217–1220.

40. Durbahn, C. M., D. L. Nuss, and D. W. Fulbright. Molecular analysis of the dsRNA associated with hypovirulence in a Michigan strain of the Chestnut blight fungus *Cryphonectria parasitica*. Phytopathology, in press. (Abstract.)

41. Elliston, J. E. 1978. Pathogenicity and sporulation in normal and diseased strains of *Endothia parasitica* in American chestnut, p. 95–100. *In* W. L. MacDonald, F. C. Cech, J. Luchok, and C. Smith (ed.), Proceedings of the American Chestnut Symposium. West Virginia University, Morgantown.

42. Elliston, J. E. 1985. Characterization of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology **75:**151–158.

43. Froehner, S. C., and K.-E. Eriksson. 1974. Induction of *Neurospora crassa* laccase with protein synthesis inhibitors. J. Bacteriol. **120:**450–457.

44. Fulbright, D. W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology **74:**722–724.

45. Fulbright, D. W., C. P. Paul, and S. W. Garrod. 1988. Hypovirulence: a natural control of chestnut blight, p. 121–138. *In* K. G. Mukerji and K. L. Garg (ed.), Biocontrol of plant diseases. CRC Press, Boca Raton, Fla.

46. Fulbright, D. W., W. H. Weidlich, K. Z. Haufler, C. S. Thomas, and C. P. Paul. 1983. Chestnut blight and recovering American chestnut trees in Michigan. Can. J. Bot. **61:**3144–3171.

47. Garrod, S. W., D. W. Fulbright, and A. V. Ravenscroft. 1985. Dissemination of virulent and hypovirulent forms of a marked strain of *Endothia parasitica* in Michigan. Phytopathology **75:**533–538.

48. Geiger, J.-P., M. Nicole, D. Nandris, and B. Rio. 1986. Root rot disease of *Hevea brasiliensis*. I. Physiological and biochemical aspects of aggression. Eur. J Pathol. **16:**22–37.

49. Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov. 1989. Coronavirus genome: prediction of putative functional domains in the non-structural polyprotein by comparative amino acid sequence analysis. Nucleic Acids Res. **17:**4847–4861.

50. Graham, T. L., and M. Y. Graham. 1991. Cellular coordination of molecular responses in plant defense. Mol. Plant Microbe Interact. **4:**415–422.

51. Grente, J. 1965. Les formes hypovirulentes d'*Endothia parasitica* et les espoirs de lutte contre le chancre du chataignier. C.R. Acad. Agric. France **51:**1033–1037.

52. Grente, J., and S. Berthelay-Sauret. 1978. Biological control of chestnut blight in France, p. 30–34. *In* W. L. MacDonald, F. C. Cech, J. Luchok, and C. Smith (ed.), Proceedings of the American Chestnut Symposium. West Virginia University, Morgantown.

53. Grente, J., and S. Sauret. 1969. L'hypovirulence exclusive phenomene original in pathologie vegetal. C.R. Acad. Sci. Ser. D **268:**2347–2350.

54. Griffin, G. J. 1986. Chestnut blight and its control. Hort. Rev. **8:**291–335.

55. Hansen, D. R., N. K. Van Alfen, K. Gillies, and N. A. Powell. 1985. Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. J. Gen. Virol. **66:**2605–2614.

56. Havir, E. A., and S. L. Anagnostakis. 1983. Oxalate production by virulent but not by hypovirulent strains of *Endothia parasitica*. Physiol. Plant Pathol. **23:**369–376.

57. Hebard, F. V., G. J. Griffin, and J. R. Elkins. 1984. Developmental histopathology of cankers incited by hypovirulent and virulent isolates of *Endothia parasitica* on susceptible and resistant chestnut trees. Phytopathology **74:**140–149.

58. Hillman, B. I., R. Shapira, and D. L. Nuss. 1990. Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* can be partially relieved by high light intensity. Phytopathology **80:**950–956.

59. Hillman, B. I., Y. Tian, P. J. Bedker, and M. P. Brown. 1992. A North American hypovirulent isolate of the chestnut blight fungus with European isolate-related dsRNA. J. Gen. Virol. **73:**681–686.

60. Hiremath, S., B. L'Hostis, S. A. Ghabrial, and R. E. Rhoads. 1986. Terminal structure of hypovirulence-associated dsRNA in the chestnut blight fungus *Endothia parasitica*. Nucleic Acids Res. **14:**9877–9896.

61. Horvath, C. M., M. A. Williams, and R. A. Lamb. 1990. Eukaryotic coupled translation of tandem cistrons: identification of the influenza B virus BM2 polypeptide. EMBO J. **9:**2639–2647.

62. Kirk, T. K., and M. Shimada. 1985. Lignin biodegradation: the microorganisms involved and the physiology and biochemistry of degradation by white-rot fungi, p. 579–605. *In* T. Higuchi (ed.), Biosynthesis and biodegradation of wood components. Academic Press, Inc., San Diego.

63. Koonin, E. V., G. H. Choi, D. L. Nuss, R. Shapira, and J. C. Carrington. 1991. Evidence for common ancestry of a chestnut blight hypovirulence-associated double-stranded RNA and a group of positive-strand RNA plant viruses. Proc. Natl. Acad. Sci. USA **88:**10647–10651.

64. Kozak, M. 1984. Selection of initiation sites by eucaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. Nucleic Acids Res. **12:**3873–3892.

65. Kräusslich, H.-G., and E. Wimmer. 1988. Viral proteinases. Annu. Rev. Biochem. **57:**701–754.

66. Lamb, C. J., M. A. Lawton, M. Dron, and R. A. Dixon. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56:**215–224.

67. Leatham, G. F., and M. A. Stahmann. 1981. Studies on laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. J. Gen. Microbiol. **125:**147–157.

68. Lemke, P. A. 1977. Fungal viruses in agriculture, p. 159–175. *In* J. A. Romberge, R. H. Foote, L. C. V. Knutson, and P. L. Lentz (ed.), Beltsville Symposium in Agriculture Research. I. Virology and agriculture. Allanhald Osmun, Montclair, N.J.

69. Leonard, T. J. 1971. Phenoloxidase activity and fruiting body formation in *Schizophyllum commune*. J. Bacteriol. **106:**162–167.

70. L'Hostis, B., S. T. Hiremath, R. E. Rhoads, and S. A. Ghabrial. 1985. Lack of sequence homology between double-stranded RNA from European and American hypovirulent strains of *Endothia parasitica*. J. Gen. Virol. **66:**351–355.

71. MacDonald, W. L., and D. W. Fulbright. 1991. Biological control of chestnut blight: use and limitation of transmissible hypovirulence. Plant Dis. **75:**656–661.

72. Marbach, I., E. Harel, and A. M. Mayer. 1985. Pectin, a second inducer for laccase production by *Botryis cinerea*. Phytochemistry **24:**2559–2561.

73. Mathews, R. E. F. 1991. Plant virology. Academic Press, Inc., San Diego.

74. Merkel, H. W. 1905. A deadly fungus on the American chestnut, p. 97–103. *In* New York Zoological Society 10th Annual

Report. New York Zoological Society, Bronx, N.Y.

75. Mittempergher, L. 1978. The present status of chestnut blight in Italy, p. 34–37. In W. L. MacDonald, F. C. Cech, J. Luchok, and C. Smith (ed.), Proceedings of the American Chestnut Symposium. West Virginia University, Morgantown.

76. Mueller, P. P., and A. G. Hinnebusch. 1986. Multiple upstream AUG codons mediate translational control of GCN4. Cell 45:201–207.

77. Newhouse, J. R. 1990. Chestnut blight. Sci. Am. 263:106–111.

78. Nuss, D. L., and Y. Koltin. 1990. Significance of dsRNA genetic elements in plant pathogenic fungi. Annu. Rev. Phytopathol. 28:37–58.

79. Oh, C.-S., and J. C. Carrington. 1989. Identification of essential residues in potyvirus proteinase HC-Pro by site-directed mutagenesis. Virology 173:692–699.

80. Paillet, F. L. 1982. The ecological significance of American chestnut [Castanea dentata (Marsh.) Borkh] in the Holocene forests of Connecticut. Bull. Torrey Bot. Club 109:457–473.

81. Paillet, F. L. 1984. Growth-form and ecology of American chestnut sprout clones in northeastern Massachusetts. Bull. Torrey Bot. Club 111:316–328.

82. Paul, C. P., and D. W. Fulbright. 1988. Double-stranded RNA molecules from Michigan hypovirulent isolates of Endothia parasitica vary in size and sequence homology. Phytopathology 78:751–755.

83. Pavari, A. 1949. Chestnut blight in Europe. Unasylva 3:8–13.

84. Powell, W. A., and N. K. Van Alfen. 1987. Differential accumulation of poly(A)$^+$ RNA between virulent and double-stranded RNA-induced hypovirulent strains of Cryphonectria (Endothia) parasitica. Mol. Cell. Biol. 7:3688–3693.

85. Powell, W. A., and N. K. Van Alfen. 1987. Two nonhomologous viruses of Cryphonectria (Endothia) parasitica reduce accumulation of specific virulence-associated polypeptides. J. Bacteriol. 169:5324–5326.

86. Racaniello, V. R., and D. Baltimore. 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science 214:916–919.

87. Rae, B. P., B. J. Hillman, J. Tartaglia, and D. L. Nuss. 1989. Characterization of double-stranded RNA genetic elements associated with biological control of chestnut blight: organization of terminal domains and identification of gene products. EMBO J. 8:657–663.

88. Rigling, D., U. Heiniger, and H. R. Hohl. 1989. Reduction of laccase activity in dsRNA-containing hypovirulent strains of Cryphonectria (Endothia) parasitica. Phytopathology 79:219–223.

89. Rigling, D., and N. K. Van Alfen. 1991. Regulation of laccase biosynthesis in the plant pathogenic fungus Cryphonectria parasitica by double-stranded RNA. J. Bacteriol. 173:8000–8003.

90. Roane, M. K., G. J. Griffin, and J. R. Elkins. 1986. Chestnut

blight, other Endothia diseases and the genus Endothia, p. 53. In APS monographs. APS Press, St. Paul, Minn.

91. Russin, J. S., and L. Shain. 1985. Disseminative fitness of Endothia parasitica containing different agents for cytoplasmic hypovirulence. Can J. Bot. 65:54–57.

92. Shapira, R., G. H. Choi, B. I. Hillman, and D. L. Nuss. 1991. The contribution of defective RNAs to the complexity of viral-encoded double-stranded RNA populations present in hypovirulent strains of the chestnut blight fungus, Cryphonectria parasitica. EMBO J. 10:741–746.

93. Shapira, R., G. H. Choi, and D. L. Nuss. 1991. Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. EMBO J. 10:731–739.

94. Shapira, R., and D. L. Nuss. 1991. Gene expression by a hypovirulence-associated virus of the chestnut blight fungus involves two papain-like protease activities. J. Biol. Chem. 266:19419–19425.

95. Siaw, M. F. E., M. Shahabuddin, S. Ballard, and J. G. Shaw. 1985. Identification of a protein covalently linked to the 5'-terminus of tobacco vein mottling virus RNA. Virology 142:134–143.

96. Snijder, E. J., J. A. Den Boon, P. J. Brendenbeek, M. C. Horzinek, R. Rijnbrand, and W. J. M. Spaan. 1990. The carboxyl-terminal part of the putative Berne virus polymerase is expressed by ribosomal frameshifting and contains sequence motifs which indicate that toro- and coronaviruses are evolutionarily related. Nucleic Acids Res. 18:4535–4542.

97. Taniaguchi, M., C. Palmieri, and C. Weissman. 1978. Qβ DNA-containing hybrid plasmids giving rise to Qβ phage formation in the bacterial host. Nature (London) 274:223–228.

98. Tartaglia, J., C. P. Paul, D. W. Fulbright, and D. L. Nuss. 1986. Structural properties of double-stranded RNAs associated with biological control of chestnut blight fungus. Proc. Natl. Acad. Sci. USA 83:9109–9113.

99. Turchetti, T. 1978. Some observations on the "hypovirulence" of chestnut blight in Italy, p. 92–94. In W. L. MacDonald, F. C. Cech, J. Luchok, and C. Smith (ed.), Proceedings of the American Chestnut Symposium. West Virginia University, Morgantown.

100. Turchetti, T. 1982. Hypovirulence in chestnut blight (Endothia parasitica) and some practical aspects. Eur. J. For. Pathol. 12:414–417.

101. Van Alfen, N. K., R. A. Jaynes, S. L. Anagnostakis, and P. R. Day. 1975. Chestnut blight: biological control by transmissible hypovirulence in Endothia parasitica. Science 189:890–891.

102. Wellink, J., and A. van Kammen. 1988. Proteases involved in the processing of viral polypeptides. Arch. Virol. 98:1–26.

103. Woodruff, J. B. 1946. Chestnut blight in Italy. Trees (J. Am. Arborculture) April:8–9, 16.